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Non-Covalent Immobilization of Quince (*Cydonia Oblonga*) Polyphenol Oxidase

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A partially purified polyphenol oxidase from quince (*Cydonia oblonga*) was immobilized on bentonite by simple adsorption at pH 6.8. The properties of the immobilized enzyme were compared to those of the free enzyme. Optimum pH and temperature were determined to be 9.0 and 45°C, respectively, showing the alteration of pH and temperature profiles by immobilization. No drastic change was observed in the K_m value after immobilization. Catechol, L-DOPA, p-cresole and pyrogallol were tested as substrates. Thermal and storage stability and reusability experiments were carried out. It was observed that the immobilized enzyme had storage stability for a period of one year but had no reusability in the batch process.

Key Words: Bentonite, polyphenol oxidase, purification, non-covalent immobilization, characterization

Introduction

Bentonite is an inexpensive matrix for enzyme protein immobilization and has been frequently utilized for this purpose. It is a clay mineral and readily available abundantly in Turkey[1]. It is also a suitable support material for polyphenol oxidase immobilization[2]. In most cases, immobilization has been carried out by covalent attachment[3−4]. Recently there has been renewed interest in immobilization by non-covalent methods[5−6] so that polyphenol oxidases, which are isolated from various sources, have been immobilized on different supports such as zeolite, sepiolite, bentonite[2], chitin[6], glass beads[7] and chitosan[8] by the non-covalent method.

Two of the most attractive properties are the simplicity of the method and the retention of activity during immobilization. Furthermore, the adsorption method is a very economical procedure for the immobilization of enzymes. The main disadvantage of this method is the weak binding of enzymes.

Immobilized polyphenol oxidases have been used to control pollution in water[9], to remove and transform toxic compounds in industrial processes[10−11], to determine catechol and other biologically active catecholamines in biologic liquids[12] as a enzymatic biosensor and to monitor the rancidification process in olive oils[13].

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In the present study quince polyphenol oxidase (EC.1.14.18.1) was immobilized on bentonite by the adsorption method. The bentonite used was obtained from the west of Turkey (Enez, Edirne). Its chemical composition is given in Table 1. The immobilized enzyme was characterized in terms of kinetic constant, optimum pH and temperature, substrate specificity, thermal and storage stability, and reusability in the batch process.

Table 1. Chemical composition of bentonite E-09

Compound	Amount %
SiO_2	70 ± 2.0
Al_2O_3	14 ± 1.0
Fe_2O_3	1.8 ± 0.2
CaO	0.7 ± 0.2
MgO	1.7 ± 0.2
K_2O	1.8 ± 0.2
Na_2O	0.4 ± 0.2

Experimental

Extraction of Polyphenol Oxidase

The extraction method previously described[14−15] was followed in a modified form. One hundred grams of quince tissue was cut quickly into thin slices and homogenized with 100 ml of 0.1 M phosphate buffer, pH 6.8 containing 10 mM ascorbic acid, 0.1% polyvinyl pirrolidone and 0.5% Triton X-100 in a Waring Blender for 3 min. The homogenate was filtered through glass wool and the filtrate was centrifuged at 160,000 x g for 30 min at 4°C using a Sanyo MS 60 Ultracentrifuge. The supernatant was brought to 30-90% $(NH_4)_2SO_4$saturation by adding $(NH_4)_2SO_4$ to precipitate proteins. The precipitated protein was separated by centrifugation at 160,000 x g for 30 min. The precipitate was dissolved in a small amount of 0.1 M phospate buffer (pH 6.8) and dialyzed at 4°C in the same buffer for 6 h replacing the buffer three times during dialysis. The dialysate was utilized as the active enzyme.

Immobilization of Quince Polyphenol Oxidase on Bentonite by Adsorption

Polyphenol oxidase was immobilized by adsorption on bentonite using an organic solvent (acetone) as an immobilization medium.

Prior to immobilization, bentonite was stirred in H_2SO_4, washed with distilled water, and dried at 105°C for 2 h.

For the immobilization 5 ml dialysate containing 0.680 mg protein/ml was mixed with 5 g bentonite under low stirring for 30 min at 4°C. After this period, 8 ml of cold acetone was added to this mixture. For the completion of adsorption, the mixture was kept at 4°C for 1 h by shaking continuously and slightly, and the final mixture was separated by vacuum filtration. Then the solid was washed with 15 ml of cold acetone and distilled water until the filtrate was free from any unbound enzyme and finally dried *in vacuo*[16]. Further experiments were carried out to select appropriate enzyme loading by using an immobilized amount of polyphenol oxidase for different amounts of support.

Determination of the Amount of Enzyme Bound on the Supports

Protein in the filtrates and dialysate were estimated by employing the method described by Lowry *et al.* using bovine serum albumine as standard[17]. Finally, the amount the protein bound on the support was found by subtracting unbound protein from total protein.

Assay of Immobilized Polyphenol Oxidase Activity

The activity of immobilized enzyme was assayed by using a modified version of the method of Burton *et al.*[18]. Twenty milligrams of biocatalyst (enzyme + support) was added to a solution of catechol (0.02 M, 5 ml) prepared in phosphate buffer (0.05 M, pH 6.8) at 25°C in a tube. The mixture was briefly but rapidly shaken to dissolve the enzyme off the support, filtered, and monitored to observe the increase in absorbance due to catechol at 420 nm for 1 min using a Shimadzu UV 160 A Spectrophotometer. The blank sample contained only 3 ml of catechol solution. Enzyme activity was calculated from the linear portion of the curve and one unit of polyphenol oxidase activity was defined as the amount of enzyme + support that caused an increase in absorbance of 0.001 per min.

Relative specific activity was calculated using the following formula:

Relative Specific Activity = Unit / mg immobilized protein

Effect of pH and Temperature

Immobilized/free polyphenol oxidase activity was assayed at various pH values using catechol as a substrate. The phosphate buffers were used between pH 5 and 10 for this purpose.

In the optimum temperature assay, immobilized/free polyphenol oxidase was incubated at temperatures ranging from 5°C to 70°C prior to measurement of its residual activity.

Determination of Kinetic Constants

K_m values of the immobilized/free polyphenol oxidase for catechol were calculated from Lineweaver-Burk plots.

Substrate Specificity of Immobilized Polyphenol Oxidase

Immobilized/free polyphenol oxidase activity was determined using four different substrates (catechol, pyrogallol, L-DOPA and p-cresole). All substrate solutions were prepared in 0.05 M phosphate buffer, pH 6.8.

Thermal and Storage Stability

Immobilized polyphenol oxidase was kept for 1 h at temperatures ranging from 10°C to 90°C before the enzyme assay. Then these activity assays were carried out using catechol as a substrate.

To determine the activity change of immobilized enzyme with time, the activity assay of immobilized preparate, which was stored in a deep freeze at -40°C, was done for a period of one year.

Reusability of Immobilized Polyphenol Oxidase

After the enzymatic activity was assayed for 50 mg of immobilized polyphenol oxidase using catechol as a substrate, this preparation was washed with cold acetone. Then the activity assays were repeated for the same enzyme preparation three times a day for three days.

Results and Discussion

Polyphenol oxidase (EC.1.14.18.1) catalyses the regioselective aerobic oxidation of monophenols to o-diphenols and their eventual dehydrogenation to o-quinons. Undesirable browning in fresh fruits and vegetables, resulting from the oxidations of polyphenolic compounds, occurs during post-harvest storage and processing[19−20]. Thus a considerable number of studies on the purification, structure and mechanisms of action of polyphenol oxidase have been performed[21]. Nisto *et al.* and Mazzei *et al.* have designed enzymatic biosensors by using immobilized polyphenol oxidase[12−22]. Wada *et al.* and Crecchio *et al.* have investigated controlling pollution in wastewater with immobilized polyphenol oxidases[10−23]. While most of these studies have been carried out with mushroom an enzymes, we have utilized an enzyme isolated from quince since it is approximately as active as that isolated from mushrooms[24]. Bentonite, as a clay mineral, has been considered a good candidate matrix for polyphenol oxidase since it is reported that it exists naturally in immobilized form in soil and participates in humification processes[2]. Furthermore, bentonite is an economical support for enzyme immobilization.

In the present study, partially purified polyphenol oxidase from quince was immobilized on bentonite by adsorption. Increasing the amount of enzyme caused a decrease in activity (Table 1). This has been explained by "crowding" of the enzyme on the surface of bentonite. At higher enzyme concentrations, the loss of activity during immobilization was about 40%. The preparation which had immobilization yield of 92% (Table 1) was used in the present work.

Table 2. Immobilization of quince polyphenol oxidase on bentonite at pH 6.8

Protein of quince PPO (mg)/5 g bentonite (x)	Protein of PPO in washing (mg) (y)	Bound protein of PPO (mg) (x-y) = A	Immobilization yield A/x . 100 = %	Relative specific activity (U/mg immobilized protein)
2.60	0.20	2.40	92.3	5221
6.20	1.25	4.95	79.8	3145
7.50	2.80	4.70	62.6	2400

The immobilized enzyme gave a K_m value of 7.6 mM as compared with 7.5 mM for the free enzyme. K_m values were 37 mM and 34 mM, respectively, for immobilized and free potato polyphenol oxidase[6]. We found that the highest activity of immobilized and free polyphenol oxidase was at pH 9.0 and pH 8.0, respectively. The activity of immobilized enzyme was higher than that of the free enzyme at alkaline pH and its pH profile was different from that of the free enzyme (Figures 1, 2). The optimum pH of immobilized mushroom polyphenol oxidase was given as 7.0 and its activity was high at alkaline pH, similar to our findings[7].

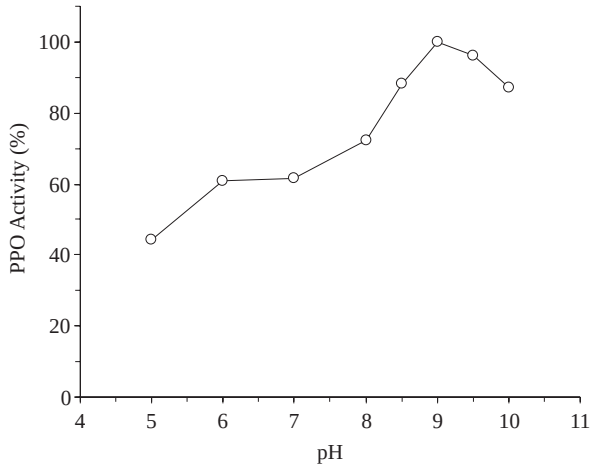

Figure 1. pH profile of immobilized enzyme

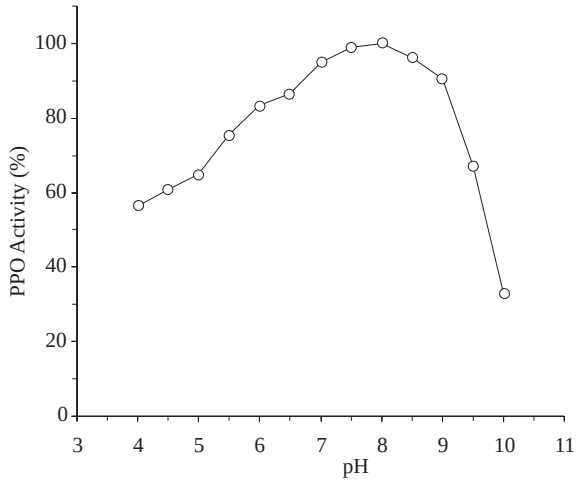

Figure 2. pH profile of free enzyme

The temperature profile of immobilized enzyme is shown in Figure 3, where it is seen that the optimum temperature is 45°C. For immobilized mushroom polyphenol oxidase, this value was 30°C[7]. The optimum temperature of free enzyme from quince is about 40°C, and has a temperature profile broader than that of immobilized enzyme (Figure 4).

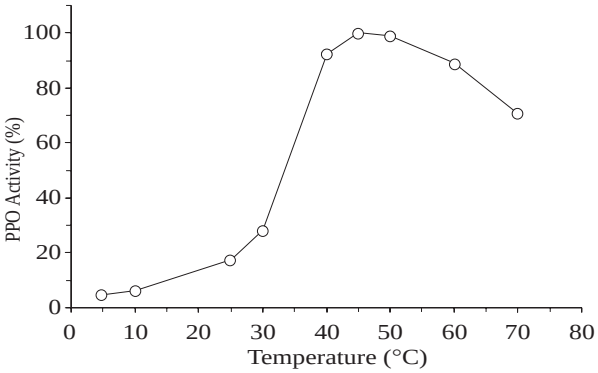

Figure 3. Temperature profile of immobilized enzyme

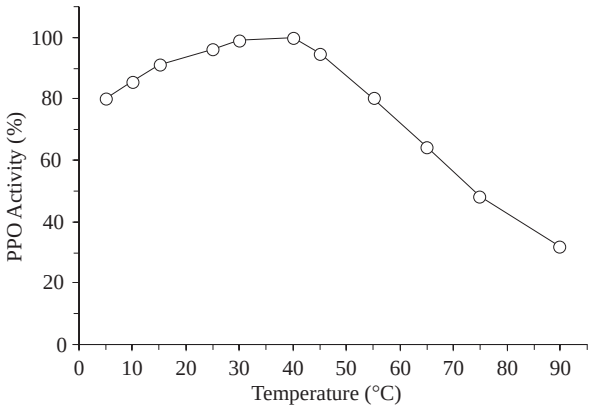

Figure 4. Temperature profile of free enzyme

Catechol, L-DOPA, p-cresole and pyrogallol were tested as substrates and the results of activity assays are summarized in Table 2. It was observed that while catalytic efficiency increased in the order catechol, p-cresole, L-DOPA, no activity was observed for pyrogallol. However, for free polyphenol oxidase, while pyrogallol is a good substrate, p-cresole is not a substrate at all, showing that immobilization may cause a change in substrate specificity (Table 2).

Table 3. Substrate specificity of immobilized and free polyphenol oxidase

Substrate	Immobilized enzyme (U/ mg immobilized protein)	Free enzyme (U/ mg protein)
Catechol	5769	2179
L-DOPA	2788	786
p-cresole	3846	NA
Pyrogallol	NA	1230

Immobilized polyphenol oxidase activity was determined at 11 different temperatures ranging from 10 to 90°C following 1 h incubation. The thermal stability profile is shown in Figure 5. Immobilized polyphenol oxidase showed almost the same activity for each temperature up to 60°C. These results indicate that immobilization protected the enzyme structure from thermal inactivity. The resistance of immobilized polyphenol oxidase to temperature is an important potential advantage for practical applications of this enzyme.

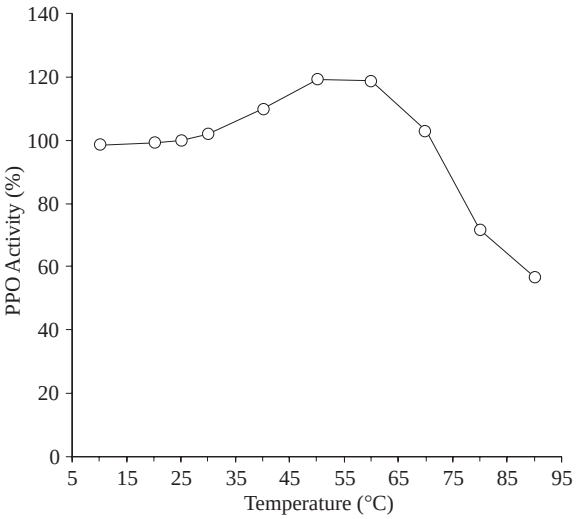

Figure 5. Thermal stability of immobilized enzyme; 20 mg of immobilized enzyme was dissolved in 1.0 mL 0.05 M phosphate buffer, pH 6.8, and was incubated at different temperatures for 30 min. It was cooled to 25°C immediately, and the activity was measured as described in the Materials and Methods

In the reusability assay of enzyme in the batch processes we determined that the percent relative conversion after the first usage decreased so markedly that the decrease in percent relative activity became greater than 10% for reaction times less than 2 h. After 4 h, this decrease was about 80% (Figure 6). These results indicated that polyphenol oxidase was being inactivated throughout its catalytic cycle. The washing and filtration of the enzyme between uses did not restore its original activity, indicating that inactivation was irreversible.

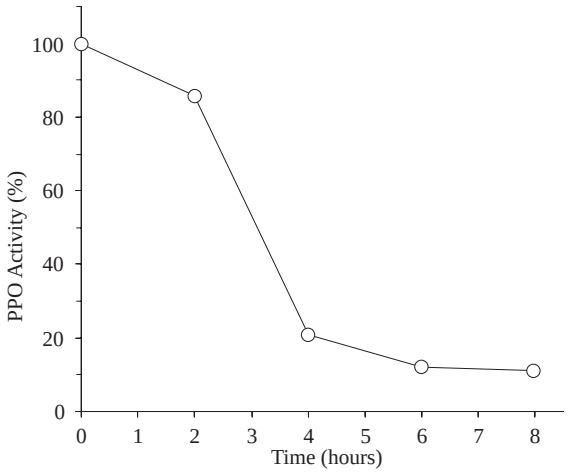

Figure 6. Reusability of immobilized enzyme

The activity assays of immobilized enzyme were carried out to determine the effect of time on the polyphenol oxidase activity for a period of one year. We observed that stability did not change considerably during this period (Figure 7). This is an advantage for industrial usage, ecological treatments and biosensor applications.

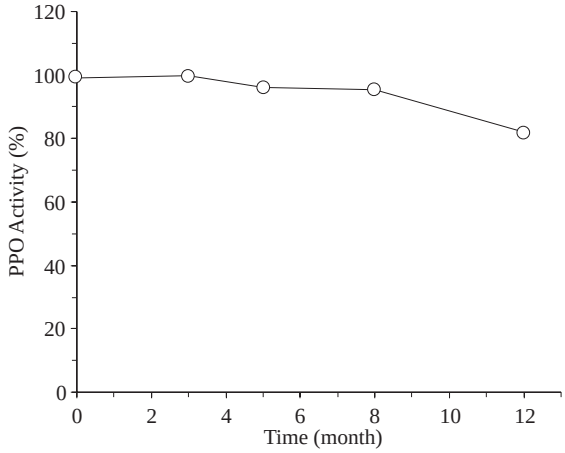

Figure 7. The storage stability profile of immobilized enzyme

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